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Abstract: Adipokines such as adiponectin and visfatin/pre-B-cell colony-enhancing factor (PBEF) have been recently shown to contribute to synovial inflammation in rheumatoid arthritis (RA). In this study, we evaluated the pathophysiological implication of visfatin/PBEF in the molecular patterns of RA synovial tissue, focusing on RA synovial fibroblasts (RASFs), key players in RA synovium. Expression of visfatin/PBEF in synovial fluid and tissue of RA patients was detected by immunoassays and immunohistochemistry. RASFs were stimulated with different concentrations of visfatin/PBEF over varying time intervals, and changes in gene expression were evaluated at the RNA and protein levels using Affymetrix array, real-time PCR, and immunoassays. The signaling pathways involved were identified. The influence of visfatin/PBEF on fibroblast motility and migration was analyzed. In RA synovium, visfatin/PBEF was predominantly expressed in the lining layer, lymphoid aggregates, and interstitial vessels. In RASFs, visfatin/PBEF induced high amounts of chemokines such as IL-8 and MCP-1, proinflammatory cytokines such as IL-6, and matrix metalloproteinases such as MMP-3. Phosphorylation of p38 MAPK was observed after visfatin/PBEF stimulation, and inhibition of p38 MAPK showed strong reduction of visfatin-induced effects. Directed as well as general fibroblast motility was increased by visfatin/PBEF-induced factors. The results of this study indicate that visfatin/PBEF is involved in synovial fibroblast activation by triggering fibroblast motility and promoting cytokine synthesis at central sites in RA synovium.

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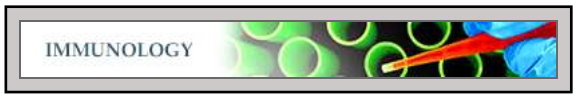

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Visfatin/Pre-B Cell Colony-Enhancing Factor (PBEF): a Proinflammatory and Cell Motility-Changing Factor in Rheumatoid Arthritis*

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* Running title: *PBEF, a proinflammatory, cell-motility changing factor in RA*

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Keywords: Fibroblasts; Cytokines; Chemokines; Rheumatoid Arthritis; Inflammation

Background: The adipokine visfatin exerts proinflammatory effects in synovial fibroblasts of patients having rheumatoid arthritis.
Results: Visfatin induces high amounts of chemokines creating a microenvironment of enhanced fibroblast motility.
Conclusion: Visfatin is therefore very capable of contributing to the inflammatory state in rheumatoid arthritis.
Significance: Enlightening visfatin pathophysiology may lead to possible therapeutic targeting in the future.

SUMMARY

Adipokines such as adiponectin or visfatin/pre-B cell colony enhancing factor (PBEF) have been recently shown to contribute to the synovial inflammation in rheumatoid arthritis (RA). In the present study, we evaluated the pathophysiological implication of visfatin/PBEF in the molecular patterns of RA synovial tissue focusing on synovial fibroblasts (SFs), key players in RA synovium. Expression of visfatin/PBEF in synovial fluid and tissue of RA patients was detected by immunoassays and immunohistochemistry. RASFs were stimulated with different concentrations of visfatin/PBEF over varying time intervals and changes in gene expression were evaluated on RNA and protein level using Affymetrix array, real-time PCR and immunoassays. Involved signaling pathways were identified. Influence of visfatin/PBEF on fibroblast motility and migration was analyzed. In RA synovium, visfatin/PBEF was predominantly expressed in the lining layer, lymphoid aggregates and interstitial vessels. In RASFs, visfatin/PBEF induced high amounts of chemokines such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), proinflammatory cytokines such as IL-6 as well as matrix-metalloproteinases like MMP-3. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) was observed after visfatin/PBEF stimulation and inhibition of p38MAPK showed strong reduction of visfatin-induced effects. Directed as well as general fibroblast motility was increased by visfatin/PBEF-induced factors. The results of the study indicate that visfatin/PBEF is involved in the synovial fibroblast activation by triggering fibroblast motility and promoting cytokine synthesis at central sites in RA synovium.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic polyarticular disease manifesting as painful inflammation of the synovial tissues and progressive destruction of the joints. Besides macrophages, T- and B-cells, synovial fibroblasts (SFs) as resident cells are key players in mediating most of the relevant pathways in this process (1, 2). Activation of RASFs in the synovium results in the production of proinflammatory cytokines and matrix-metallo-proteinases (MMPs). RASFs actively perpetuate inflammation as well as matrix degradation and invasion leading to progressive destruction of the articular cartilage and the adjacent bone, thus determining the outcome of the disease (1, 3, 4).

The terms adipocytokines or adipokines are used for cytokine-like molecules synthesized by adipocytes, e.g. including adiponectin, leptin, resistin and visfatin. There is growing evidence that adipose tissue is not only a repository for triglycerides or a passive connective tissue, but in fact an active endocrine organ regulating energy homeostasis and metabolism (5). Moreover, adipose tissue and obesity are connected with a low-grade state of inflammation and play a role in other chronic inflammatory disorders including RA (6, 7).

The expression of the adipokine pre-B-cell-colony-enhancing factor (PBEF) in plasma, synovial fluid as well as in the inflamed synovium of RA patients is elevated (8-11). In experimental settings like antigen-induced arthritis in mice similar results could be seen (8). Thus far, it has been shown that during activation of immune cells such as macrophages, monocytes, dendritic cells, neutrophils and T- and B-cells, visfatin/PBEF expression is increased (12-17). In turn, it has been reported that visfatin/PBEF up-regulates IL-ıβ IL-1ra, IL-6, IL-10 and TNF-α in PBMCs and IL-1β, IL-6 and TNF-α in $CD14^+$ monocytes (12). It is known that visfatin/PBEF induces different intracellular signaling pathways, e.g. AP-1 and NF-κB in RASFs (9). In $CD14^+$ monocytes, the visfatin/PBEF-induced cytokine production could be reduced by inhibiting p38MAPK- and MEK1-pathways (12). Given the fact, that visfatin/PBEF acts additionally as the rate-limiting enzyme in the salvage pathway of NAD, recycling NAD from nicotinamide, it prevents neutrophil apoptosis in experimental inflammation and clinical sepsis and promotes vascular smooth muscle cell maturation thus extending their lifespan (13, 18, 19).

Visfatin/PBEF was originally described as a cytokine involved in early B-cell development and was later renamed visfatin due to the fact that it is mainly secreted by visceral fat (20, 21) Recent studies showed that visfatin/PBEF is up-regulated in activated RASFs under inflammatory stimuli such as STAT-3-dependent IL-6 trans-signaling on the one hand and on the other by poly(I-C)-mediated TLR-3 activation (8, 9). In turn, visfatin/PBEF induces IL-6 in RASFs suggesting a positive feedback mechanism due to proinflammatory activities of this protein (9).

In our study, we investigated the effects of visfatin/PBEF on RASFs in detail with the main focus specifically on the change in gene and protein expression mediated by visfatin/PBEF, signaling pathways involved in those processes and alterations in fibroblast motility in order to further clarify the role of visfatin/PBEF in RA.

EXPERIMENTAL PROCEDURES

Tissue specimens and cell culture

During routine synovectomy synovial tissue was obtained from patients with RA and osteoarthritis (OA). Patients met the 1987 criteria of the American College of Rheumatology (ACR) classifying RA and OA (22, 23). RASFs were cultured as described recently (24-26). Synovial fluid of RA patients with articular effusion was aspirated. Primary human lymphocytes were isolated with Ficoll-gradient centrifugation from buffy coat samples of healthy donors. Primary human lymphocytes were cultured in RPMI 1640 (PAA Laboratories) supplemented with 5% human serum (PAA Laboratories), 100 U/ml penicillin, 10 µg/ml streptomycin and 10 mM HEPES (PAA Laboratories) at 37°C and 5% CO_2.

Stimulation assays

Recombinant human visfatin/PBEF (100ng/ml, subsequently mentioned as visfatin/PBEF, Biovendor) was used throughout the experiments if not mentioned otherwise. To exclude effects of stimulation-related cell division, cell counting was performed.

Dose-effect relationship

RASFs were stimulated with increasing concentrations of visfatin/PBEF (2.5-, 5-, 10-, 50-, 100-, 250-, 500-, 2500-, 5000-, 10000ng/ml). Unstimulated RASF served as control, whereas stimulation with adiponectin (25µg/ml, R&D Systems) served as positive control (27). IL-6 and -8 served as parameters.

Time-dependent response

RASFs were stimulated with visfatin/PBEF for 4h up to 48h. IL-6 and -8 production was quantified over time.

Affymetrix GeneChip® expression analysis

RASFs were stimulated with 500ng/ml visfatin/PBEF for 15h. RNA was extracted using the RNeasy™ MiniPrep Kit (Qiagen). Target preparation and hybridization for the Affymetrix human genome U133 Plus 2.0 GeneChip® (AFFX) was performed according to the protocol. Results of the two condition design were analyzed with the GeneSpring microarray analysis software (Silicon Genetics) to obtain Increase/Decrease or No Change calls.

Real-time PCR

RNA was isolated using the RNeasy™ Mini Kit (Qiagen). Reverse transcription was performed using AMV Reverse Transcriptase (Promega) and random hexamer primers (Roche). Primers (listed in Supplemental data 1) were designed and efficiency for each primer pair was tested using standard curve method ($E=10^{-1/slope}$) considering 2.00±0.05 acceptable for experiments. Real-time PCR was performed using LightCycler® (Roche) with SYBR Green I (Roche) as detection system. Melting curve analysis was used to confirm specificity of amplification. 18S RNA served as reference gene. Results were analyzed using Roche LightCycler® software.

Western Blot analysis of p38 phosphorylation

Cell extracts were harvested in NETN buffer (0.5% NP-40, 1mM EDTA, 20mM Tris-HCl at pH 8.0, 100mM NaCl and 10% glycerol) containing 1x complete protease inhibitor cocktail (Roche). Protein was separated on a SDS 10% (w/v) polyacrylamide gel and blotted onto nitrocellulose membrane (BioRad). Proteins were detected by antibodies against phosphorylated and total p38 (Cell Signalling Technology). Detection was performed by horseradish peroxidase-conjugated secondary antibodies (Dako) and an enhanced chemiluminescence detection kit (ECL^+ Western Blotting Detection System, GE Healthcare). All western blots were probed for Cyclophilin B (Abcam) to ensure equal loading of samples.

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISAs and EIAs (R&D Systems; Phoenix Europe) were performed in accordance with the protocol. Absorption was measured at 450nm and data were analyzed using Magellan software (Tecan).

Inhibition of signal transduction pathways

Cultured RASFs were preincubated for 1.5h with chemical inhibitors of signal transduction pathways: 1) p38MAPK-inhibitor SB203580 (20μM; Sigma-Aldrich), 2) cell-permeable myristoylated protein kinase C (PKC) inhibitor 20-28 (40μM; Calbiochem) 3) cell-permeable myristoylated protein kinase A (PKA) inhibitor 14-22 (2μM; Calbiochem), 4) cell-permeable NF-κB activation inhibitor (40μM; Calbiochem). Subsequent to the preincubation, cells were stimulated with visfatin/PBEF for 6 and 15h in presence of inhibitors. Stimulation of RASFs with visfatin/PBEF served as positive control, unstimulated RASFs with or without inhibitors as negative control.

Immunohistochemistry

Snap frozen RA and OA synovial tissues sections were prepared. Nonspecific binding was blocked with 2% bovine serum albumin followed by overnight incubation in a moist chamber at 4°C with rabbit anti-human visfatin antibodies (10μg/ml; Bethyl Laboratories (9)). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 100% methanol. Sections were then incubated with Histofine Simple Stain MAX PO (multi) anti-mouse/-rabbit (Nichirei Biosciences) for 30 minutes. Color development with AEC-substrate (Vector Laboratories) at room temperature was stopped after microscopic examination followed by counterstaining of the nuclei using hematoxylin. Rabbit isotype-matched IgG sera (Santa Cruz Biotechnology) served as isotype control, mouse anti-human vimentin (Dako) as positive control. Negative control without primary antibody was carried out.

Chemotaxis

Migration of RASFs was measured using a 48-well Boyden microchemotaxis chamber (Neuroprobe). Conditioned medium was prepared incubating RASFs for 16h in DMEM without FCS. In contrast, stimulated media were acquired by stimulation for 16h with: 1. visfatin/PBEF (100ng/ml), 2. visfatin/PBEF and p38MAPK inhibitor (100ng/ml; 20μM) and 3. p38MAPK inhibitor (20μM); all in DMEM without FCS. Besides using different cell populations, experimental replicates were also performed.

20,000 cells in 50μl conditioned medium were placed in the upper chamber. In the lower chamber, 30μl of conditioned medium (migration baseline), medium with 10% FCS and without FCS (positive and negative controls, respectively) and stimulated media (as mentioned above) were added. Both chambers were separated by an 8μm pore-size filter (GE Osmonics). After migration for 7h, cells on the

upper filter side were scraped off. Filters were fixed and stained. Of each well, 6 areas were photographed, amount of cells was counted, and data were expressed as migration index (ratio of sample to baseline, (28)).

Migration of lymphocytes was measured using a 24-well Transwell® migration assay (Corning). A total of 10^6cells was added onto the filter. Into the lower well the prepared conditioned medium and stimulated media (as described above) were placed. In the control experiment, we tested visfatin/PBEF diluted in normal DMEM and 10% FCS. Cells were allowed to migrate for 4h at 37°C. Cells in the lower well were enumerated with a haemocytometer.

Scrape motility assay

RASFs were preincubated with increasing concentrations of visfatin/PBEF (5-, 25-, 50-, 100-, 250ng/ml) for 1.5h. Beginning from the centre of each well, cells were removed by scraping using a 100µl pipette tip. To visualize cell motility within the scrape line, photos of the defined, cell-free scraped areas were taken every 1.5 hours between 8 to 17h after scraping (29). Cells migrating into the gap were counted and data were expressed as comparison of sample to baseline in percentages. Besides using different cell populations, experimental replicates were performed as well.

Statistics

Statistical analysis was performed using Student *t* test. Arithmetic means and standard errors of the mean (SEM) were calculated. Issues were regarded as significant for $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Statistical evaluation was performed using GraphPad Prism 5.

RESULTS

Detection of Visfatin/PBEF in RA synovial fluid and synovium

The mean concentration of visfatin/PBEF in RA synovial fluid was 76.26±7.22ng/ml (n=24; Figure 1A). A strong expression of visfatin/PBEF in RA synovium (n=3), mainly in the synovial lining layer, in lymphoid aggregates and perivascular areas could be observed indicating contribution to the inflammation of the synovium by local visfatin/PBEF production independent of adipose tissue. Visfatin/PBEF protein was expressed in OA synovium (n=2) as well but to a lesser extent and mainly in regions of interstitial vessels (Figure 1B).

Identification and confirmation of genes regulated by visfatin/PBEF in RASFs

To elucidate the effects of visfatin/PBEF on RASFs in a broad approach, the changes in gene expression and protein production were analyzed using AFFX (n=1) as screening method, and real-time PCR (n=7-9) and ELISA (n=4-5) for confirmation. Of note, the fold induction was different as expected between the non-quantitative AFFX in comparison to the quantitative techniques real-time PCR and ELISA as well as due to the different stimulation concentrations (500ng/ml versus 100ng/ml).

Several genes were strongly altered by visfatin/PBEF (Table 1). Amongst them, chemokines of the CXC- and CC-families were predominantly represented and showed the highest fold changes. Chemokines (CXCL-1, -2, -3, -5, -6, -8 and CCL-2, -5, -13, -20) were therefore quantified on RNA as well as on protein level and their regulation could be confirmed in each case (see Table 1). Verification of the observed regulation was also performed on the protein level for IL-6 and MMP-3. In addition, several cytokines including interleukin-1β, -6, -7, -8, -15, -32, -33, interleukin 1 receptor antagonist, as well as the adipokines adiponectin and PBEF itself were induced in the AFFX (see Supplemental data 2). A variety of key factors in RA pathophysiology were not regulated on protein level in RASFs by visfatin/PBEF: pro-MMP-1, activin A, OPG, IGFBP-2, -3, TIMP-1, -2 (n=3-4; Supplemental data 3). IL-1β and TNF-α levels were below the detection level (data not shown).

Establishment of dose-dependent relationships and time-dependent responses for visfatin/PBEF stimulation of RASFs

With regard to the variability of visfatin/PBEF concentrations in synovial fluids of RA patients, we determined whether the concentration of visfatin/PBEF plays a critical role in the production of proinflammatory markers like IL-6 and IL-8. We further investigated whether the incubation period of RASFs with visfatin/PBEF leads to an additional increase or suppression of cytokine production over time.

Dose-dependent relationships

The effect of visfatin/PBEF on IL-6 and IL-8 production of RASFs was dose-dependent. Basal concentration of IL-6 was 23.3pg/10³cells, which was increased up to 477.96pg/10³cells by visfatin/PBEF stimulation (100ng/ml). IL-8 was increased from 0.72pg/10³cells up to 199.49pg/10^3cells (Figure 2A). For subsequent stimulation experiments 100ng/ml visfatin/PBEF was used, which was located in the linearly increasing section of the curve and showed a significant effect on the production of proinflammatory cytokines. Further increase of

visfatin/PBEF concentration did not result in an additional increase in cytokine production and lead to a plateau in the dose-dependency curve (Figure 2A).

Time-dependent responses

After stimulating RASFs for 15h with visfatin/PBEF (100ng/ml), the basal concentration of IL-6 was increased from 14.9pg/10^3cells up to 106.04pg/10^3cells. For IL-8, the basal concentration was increased from 3.97pg/10^3cells up to 67.42pg/10^3cells. Extending the incubation period up to 48h resulted in reduction of cytokine production maybe due to counterregulation (Figure 2B). However, 15h of incubation were used throughout the stimulation experiments to avoid secondary regulatory mechanisms taking place after longer incubation periods *in vitro*.

Signaling pathways involved in visfatin/PBEF-mediated production of IL-6 and CCL-2

Inhibition of the intracellular pathways of p38MAPK (12, 30), NF-κB (9), PKA (31) and PKC (32, 33) in RASFs was performed to observe whether the induction of proinflammatory cytokines (i.e. IL-6) and chemokines (i.e. CCL-2) by visfatin/PBEF depend on them.

IL-6

Incubation with the inhibitor of the p38MAPK pathway resulted in strong reduction of visfatin/PBEF-mediated IL-6 production in RASFs. IL-6 production was reduced from 439.3±103.09pg/10^3cells to 150.4±50.31pg/10^3cells (reduction of 66%; p=0.033; n=3; Figure 3A) after 15h and from 88.16±22.17pg/10^3cells to 24.47±5.29pg/10^3cells (reduction of 72%; p=0.087; n=3; Figure 3B) after 6h compared to visfatin/PBEF-stimulated RASFs without p38MAPK inhibitor. Other inhibitors (PKC inhibitor 20-28, PKA inhibitor 14-22, NK-κB activation inhibitor) had no influence on IL-6 production (Supplemental data 4).

CCL-2

None of the inhibiting factors significantly reduced visfatin/PBEF-mediated CCL-2 production in RASFs in contrast to IL-6 production (Supplemental data 4).

p38 MAPK

Furthermore, we observed a clearly elevated basal phosphorylation of p38 in RASF as exposure to visfatin/PBEF (100ng/ml) for 5 minutes strongly activated the p38-signaling cascade (Figure 3C).

Visfatin/PBEF influences synovial fibroblast and lymphocyte motility

Next, we analyzed whether the induction of pro-inflammatory factors and chemokines induced in RASFs *via* visfatin/PBEF leads to a cytokine environment of increased directed (*chemotaxis assay*) and/or general (*scrape assay*) cell motility of RASFs and/or lymphocytes.

Scrape assay

The aim was to determine whether increasing concentrations of visfatin/PBEF induce cell motility not directed towards a chemokine gradient. The contribution of cell proliferation to the repopulation of the scraped zone was minimized using serum-free media. The findings demonstrated augmented cell motility for concentrations of visfatin/PBEF of 5, 25 and 50ng/ml (n=3; Figure 4B-D). The strongest increase in motility was found for visfatin/PBEF at 25 ng/ml (+13.3±2.7%, p=0.038 and +18.4±3.6, p=0.035 compared to baseline after 8 and 15.5h; Figure 4C). No significant change in fibroblast motility was discovered for visfatin/PBEF at 100 and 250ng/ml (data not shown). The effects were evened out at the latest time points due to completely filling in the scraped gap. Interestingly, visfatin/PBEF at 100ng/ml, the concentration used for the chemotaxis experiments, showed almost no altered general cell motility.

Chemotaxis assay

Stimulation of RASFs with visfatin/PBEF led to increased amounts of chemokines (Table 1). Migration towards these media could be detected analyzing RASFs and lymphocytes. The migration index for RASFs was 2.73±0.45 (n=2; Figure 5A). Supplementary experiments showed that migration to visfatin/PBEF-stimulated media could nearly be reduced to baseline (1.08±0.59; n=2; 60% reduction) if preincubated with the p38MAPK inhibitor. The p38MAPK inhibitor itself reduced to some extent the migratory potential of RASFs (0.39±0.31; n=2; Figure 5A). Simultaneous to the effects of visfatin/PBEF-induced factors on RASFs, lymphocyte migration was increased, but could be reduced *via* p38MAPK inhibition (n=2; Figure 5B). To see, whether increased migration is indeed caused by visfatin/PBEF-induced factors and not by visfatin/PBEF itself, we measured lymphocyte migration towards media containing 100ng/ml visfatin/PBEF and found no significant change in migration (n=2; Figure 5C).

DISCUSSION

In the present study, we investigated the potential of visfatin/PBEF to act as an effector molecule in RA. The significant changes in gene expression of RASFs mediated by visfatin/PBEF, especially the induction of a variety of chemokines and moreover proinflammatory and matrix-degrading factors, could be confirmed on RNA and protein level. This supports the hypothesis that visfatin/PBEF creates an inflammatory molecular environment of increased fibroblast and leukocyte motility within RA synovial tissue.

Inflammation of the synovium is a hallmark in RA and activated RASFs play a central role in local pathophysiological mechanisms. In the inflamed synovium of RA patients accumulation of visfatin/PBEF was most dominant at the site of cartilage invasion (9), in the lining layer, in lymphoid aggregates and around interstitial vessels, confirming that fibroblasts, lymphocytes and endothelial cells are expressing visfatin/PBEF in the synovial tissue and that are exposed to increased concentrations of visfatin/PBEF in return (8, 20, 34).

The lining layer contains high numbers of activated RASFs characterized by a high basal production of IL-6 and MMPs advancing chronic inflammatory responses, contributing to T-cell and B-cell activation and progressive cartilage destruction (35). Visfatin/PBEF enhances these effects by increasing the production of IL-6 and MMP-3, -10, -12 and -19. Therefore, visfatin/PBEF intensifies the aggressive phenotype of RASFs. This holds also true for visfatin-mediated stimulation of chondrocytes, resulting in higher amounts of prostaglandin E2 and MMP-3, and CD^+ 14 monocytes, expressing higher amounts of IL-6, IL-1β and TNF-α, as recently reported (12, 36). By elucidating the change in gene expression of RASFs after visfatin/PBEF stimulation, we could show that a broad variety of chemokines of both, the CXC- and the CC-cluster, are strongly up-regulated suggesting that visfatin/PBEF mediates chemoattraction in RA synovium to a significant extent. In addition, visfatin/PBEF elevates the expression of adhesion molecules like VCAM-1, ICAM-1 and -2 enabling RASFs to increased attachment to cartilage but also to contribute to cell migration (1). Chemoattraction is mediated via enhanced cell motility. Our results support the idea that visfatin/PBEF operates as an cell motility increasing molecule for RASFs in vitro in a similar way as it was shown for $CD14^+$ monocytes and $CD19^+$ B cells under other pathophysiological conditions (12). The visfatin/PBEF-induced cytokine-environment exerts chemoattractive properties promoting directed fibroblast motility to the site of visfatin/PBEF expression. Furthermore, the visfatin/PBEF-induced cytokine-environment contributes to lymphocyte recruitment. Therefore, visfatin/PBEF may contribute to accumulation of RASFs in the lining layer as well as at sites of cartilage invasion (1, 37) resulting in increased matrix-destruction and -remodeling at the invasion zone, a characteristic of hyperplastic RA synovium.

The inflammatory process in RA synovium is also driven by the influx of inflammatory cells into the synovial tissue and activated interstitial vessels play a key role in the extravasation of leukocytes. Increased expression of visfatin/PBEF around interstitial vessels and a strong induction of IL-8, VEGF and ECGF, factors with strong pro-angiogenic effect (38, 39), was observed. It is likely that visfatin/PBEF promotes also RA synovial angiogenesis to some extent. This process has been demonstrated for visfatin/PBEF-mediated activation of ERK-1/-2 pathways as well as for increase of FGF-2 in human endothelial cells (40-43).

Due to the fact that IL-6 and MMP-1 production is closely linked to PKC and p38MAPK activation (30-32, 44-46) and that initiation of common pathways such as PKA and NF-κB result in inflammatory responses of RASFs (30, 33), we investigated whether the observed effects were mediated *via* those pathways. Although it was shown that visfatin/PBEF activates the NF-κB pathway in RASFs (9), the only significant effects we could detect were seen by inhibition of the p38MAPK pathway. Of interest, p38MAPK inhibition in animal models of arthritis leads to improvement of the disease severity, thus reflecting its central role in arthritis (47, 48).

Taken together, the results of the study show that visfatin/PBEF is a strong effector molecule in the pathophysiology of RA, specifically by contributing to the production of proinflammatory chemokines in RASFs, matrix-degrading enzymes and proangiogenic molecules. The data also support the idea that the effects of adipokines are not restricted to adipose tissue but are operative in chronic inflammatory joint diseases.

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FOOTNOTES

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#**Competing interests** none

¶**Ethical approval** Written informed consent to use synovium or synovial fluid for research purposes was obtained from each patient. The local ethic commission of the Justus-Liebig-University, Gießen approved the study.

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[5]The abbreviations used are: PBEF, Pre-B cell colony-enhancing factor; RA, rheumatoid arthritis; SF, synovial fibroblast; MAPK, mitogen-activated protein kinase; IL, interleukin; MCP, monocyte chemotactic protein; MMP, matrix-metallo proteinase; PKC, protein kinase C; PKA, protein kinase A; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; SEM, standard error of the mean; AFFX, Affymetrix array

FIGURE LEGENDS

FIGURE 1. Detection of visfatin/PBEF in RA synovial fluid and synovium. **A**, Mean concentration of visfatin/PBEF in the synovial fluid of RA patients (n=24; age: 66.1±16 years; 83.3% female) measured by ELISA. **B**, Nuclei-counterstained immunohistochemical analysis for visfatin/PBEF protein in RA (n=3) and OA synovium (n=2). Visfatin/PBEF protein shows red, nuclei blue (magnification x400). In RA synovium, SFs in the lining layer showed a strong expression of visfatin/PBEF (black arrow). Small interstitial vessels (black arrowhead) and lymphoid aggregates (white arrow) express visfatin/PBEF protein as well. In OA synovium, minor vessels of the interstitium show visfatin/PBEF protein expression (white arrowhead). Isotype control using OA synovium was performed. ss, synovial space; ll, lining layer; sl, sublining; v, vessel; la, lymphoid aggregate; i, interstitium.

FIGURE 2. Dose-dependent relationship and time-dependent response after visfatin/PBEF stimulation of RASFs. **A**, Top line. RASFs were incubated for 15h with increasing concentrations of visfatin/PBEF (presented range 2.5 to 2500 ng/ml). IL-6 and IL-8 production was measured by ELISA. Data is expressed as concentration of cytokine (pg/10^3cells) to stimulation concentrations of visfatin/PBEF. **B**, Bottom line. RASFs were stimulated with 100 ng/ml visfatin/PBEF for 4 up to 48 hours. IL-6 and IL-8 production was measured by ELISA. Data is presented as concentration of cytokine (pg/10^3cells) to stimulation time. Dotted lines display the stimulation concentration and time chosen for subsequent experiments.

FIGURE 3. Involvement of the p38 MAPK pathways in visfatin/PBEF-mediated production of IL-6 in RASFs using an intracellular signaling inhibitor. RASFs (n=3) were stimulated with or without visfatin/PBEF (100 ng/ml) together with and without the chemical inhibitor of p38MAPK. Values are expressed as arithmetic means ± SEM. **A**, after 15 hours. **B**, after 6 hours. **C**, Basal and visfatin/PBEF induced activation of phospho-p38 in RASF. * $p<0.05$.

FIGURE 4. Visfatin/PBEF influences synovial fibroblast motility. **A,** Scrape assay pictures represent the difference in cell motility over time between cell culture wells of the negative control and wells of the sample (visfatin/PBEF 25ng/ml). Pictures from the start of the experiment and after 8 hours and

15.5 hours are displayed. Scrape assays were performed as described in methods (n=3). Results were expressed as comparison of samples to baseline (percentages). The graphs show enhanced cell motility in the samples after 8h and 15.5h for **B**, 5 ng/ml; **C**, 25 ng/ml; **D**, 50 ng/ml. * $p<0.05$.

FIGURE 5. Visfatin/PBEF influences synovial fibroblast motility. **A**, RASF chemotaxis assays were performed (see methods) and migration index was calculated (baseline set to 1, ratio of sample to baseline). Means ± SEM are displayed as bars (n=2). **B**, Demonstrating lymphocyte migration to conditioned media of RASF (n=2, total number of migrated lymphocytes are shown), results are expressed as means ± SEM (see methods). **C**, Incubation with 100 ng/ml visfatin/PBEF had only a slight effect on lymphocyte migration (n=2, fold increase of migration is shown).

TABLES

Table 1 Change in the gene expression of RASFs after stimulation with visfatin/PBEF: Selection of genes regulated by visfatin/PBEF

Gene Name	Gene Symbol	Fold Change AFFX	Fold Change Real-Time	Fold Change ELISA
Chemokines				
chemokine (C-C motif) ligand 20 (CCL20)	MIP-3α	1024.00		28.97±11.5
chemokine (C-C motif) ligand 5 (CCL5)	RANTES	473.71		30.56±13.79
chemokine (C-X-C motif) ligand 8 (CXCL8)	IL-8	164.03		51.29±13.79*
chemokine (C-X-C motif) ligand 1 (CXCL-1)	GRO-α	90.51	17.21±4.69**	18.28±8.05
chemokine (C-X-C motif) ligand 3 (CXCL3)	GRO-γ	51.98	14.77±2.82**	
chemokine (C-X-C motif) ligand 5 (CXCL5)	ENA-78	22.63	14.32±2.79**	6.39±1.22*
chemokine (C-X-C motif) ligand 6 (CXCL6)	GCP-2	18.38	114.82±35.6*	12.88±3.26*
chemokine (C-X-C motif) ligand 2 (CXCL2)	GRO-β	17.96	10.95±2.72**	
chemokine (C-C motif) ligand 13 (CCL13)	MCP-4	13.93	6.96±1.49**	
chemokine (C-C motif) ligand 2 (CCL2)	MCP-1	13.93	4.83±1.37*	3.29±0.95
Cytokines				
interleukin 6	IL6	10.56		6.07±0.94*
Proteinases & Peptidases				
matrix metallopeptidase 3	MMP3	59.71		3.63±0.77*
matrix metallopeptidase 1	MMP1	16.00		1.19±0.14

Data are presented as mean value ± standard error of the mean; * = <0.05; ** = <0.01

FIGURE 1

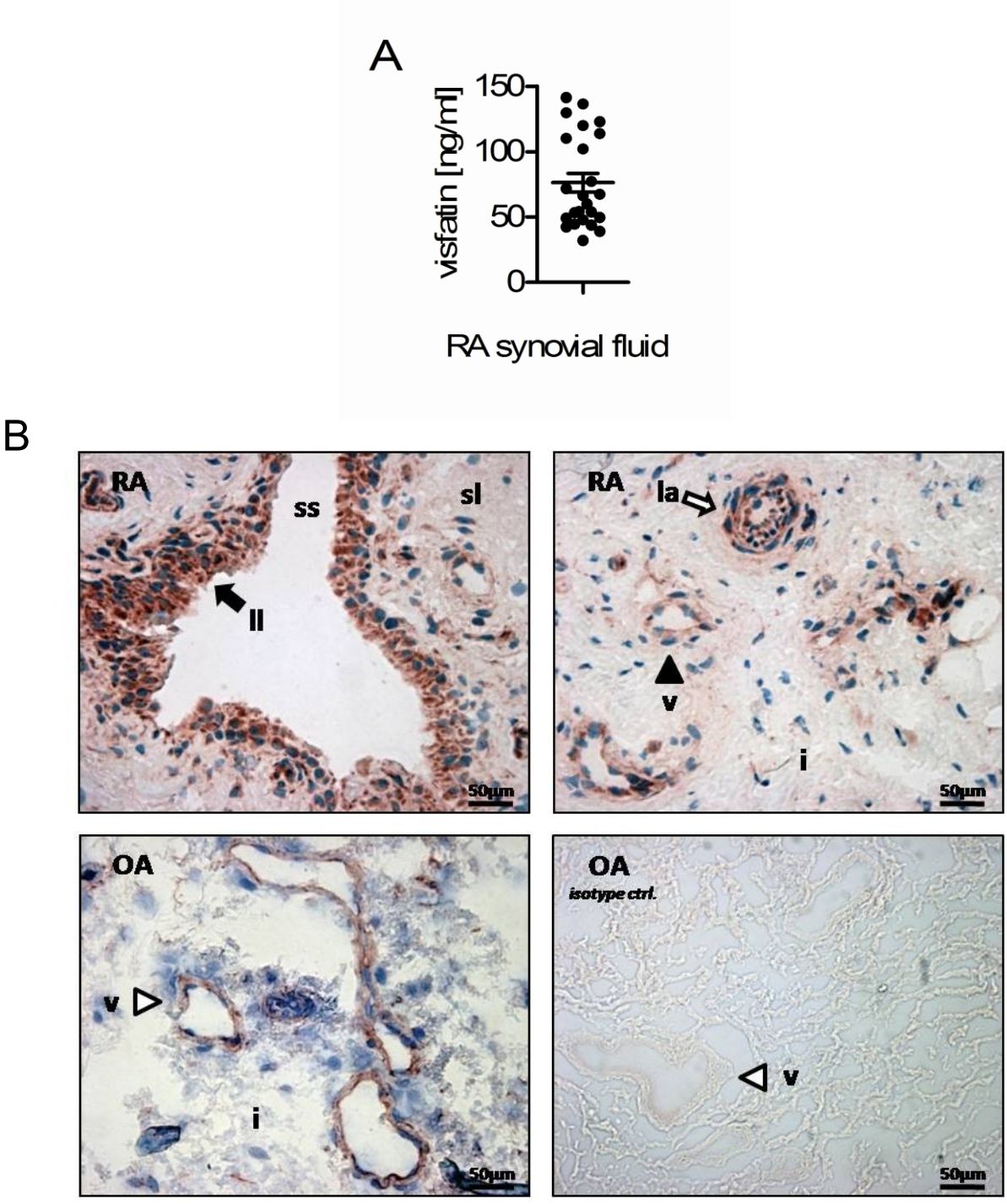

FIGURE 2

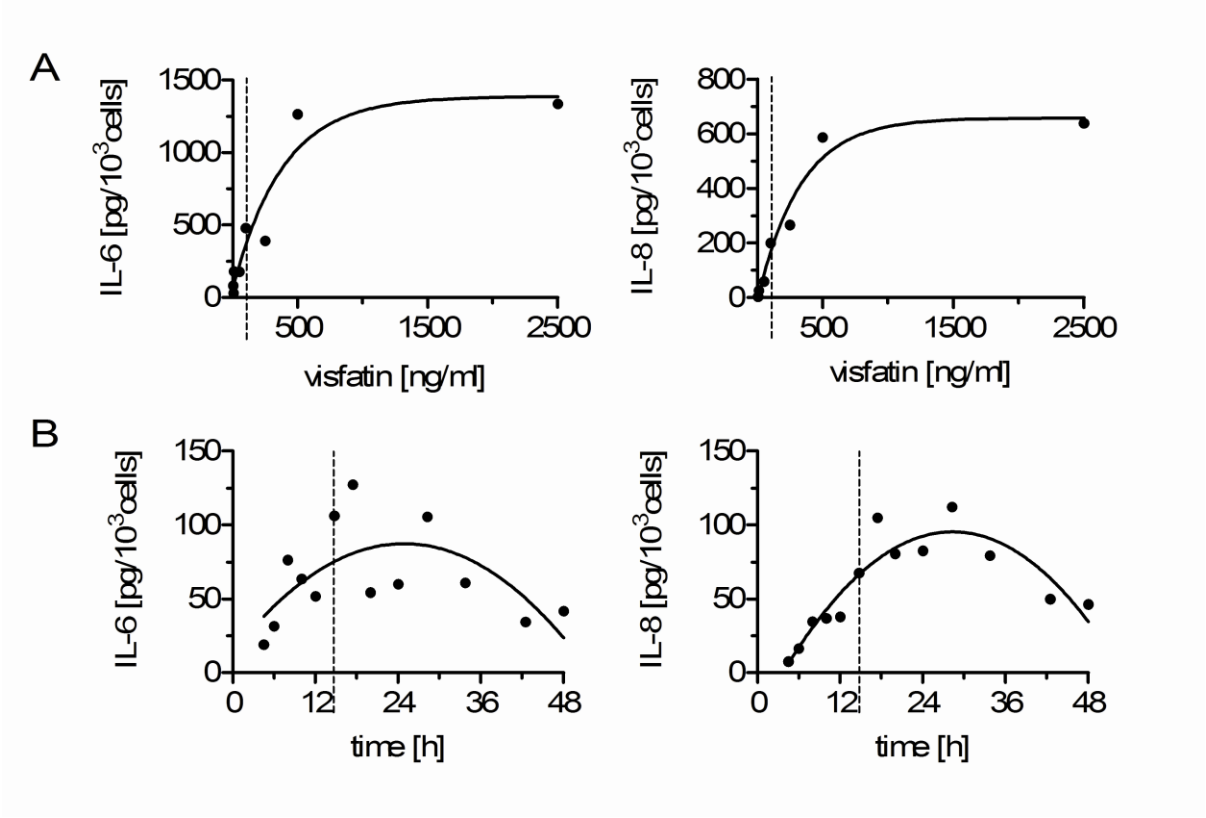

FIGURE 3

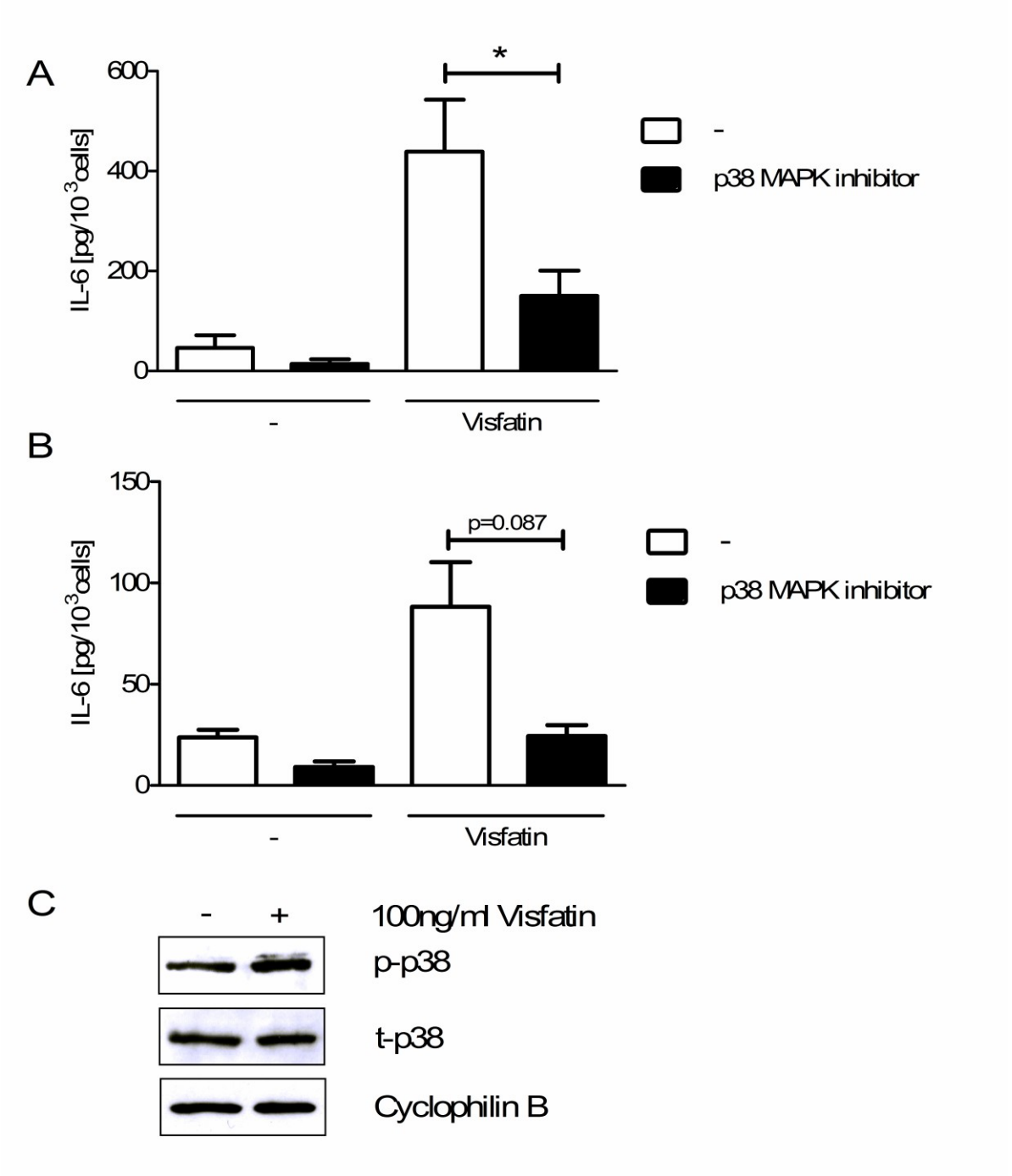

FIGURE 4

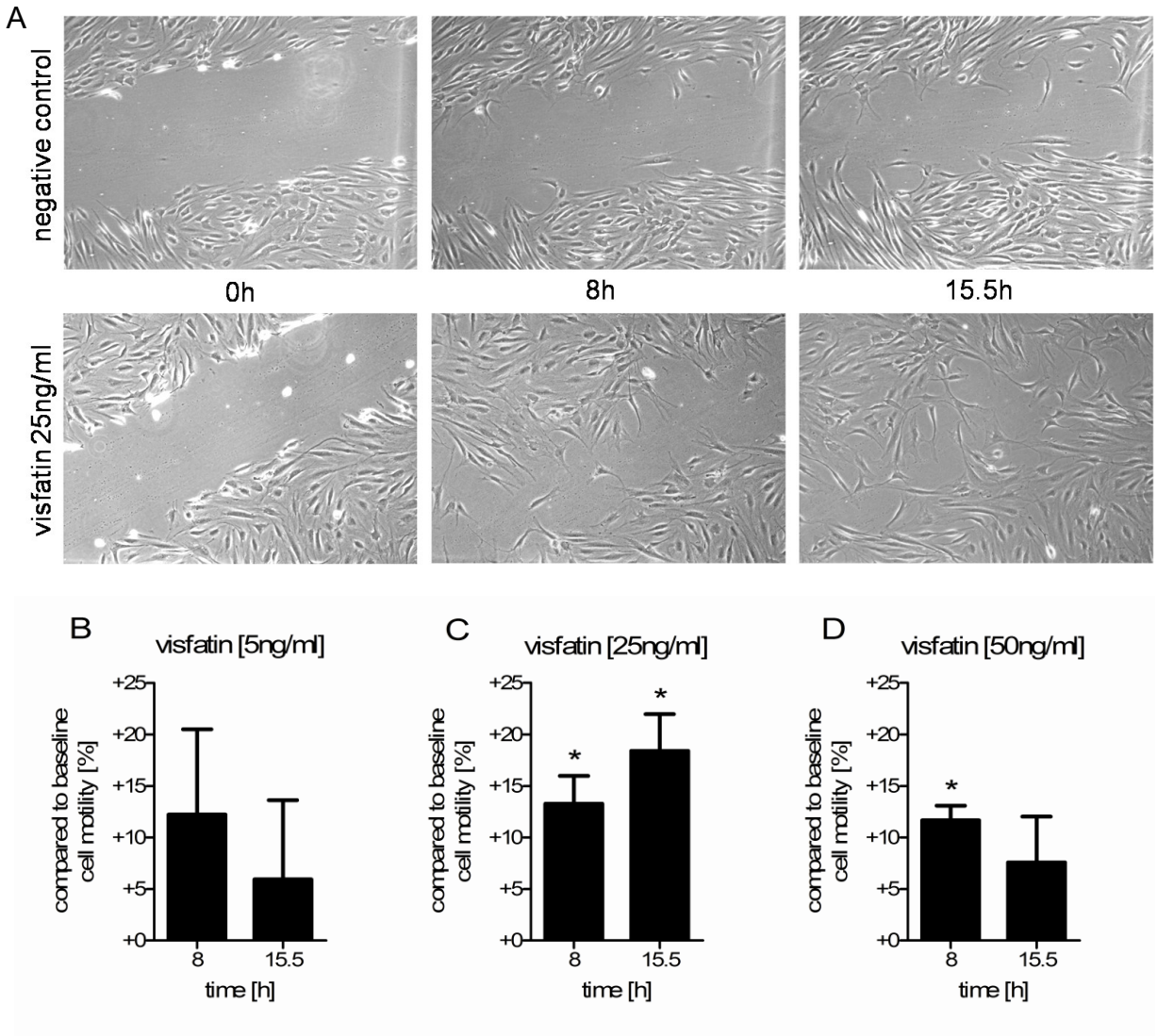

FIGURE 5